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Application of capillary zone electrophoresis to the analysis of betalains from *Beta vulgaris*

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Abstract

Capillary zone electrophoresis (CZE) with multi-wavelength detection was used for the separation of the main betacyanins, betanin and isobetanin, and the corresponding aglycones from extracts of *Beta vulgaris*. Optimum separation was achieved with a fused-silica capillary tube and a citric acid–Na_2HPO_4 buffer (10 m*M*). The applied voltage was −22 kV and the capillary thermostating temperature was kept constant at 15°C. The results of the quantitative CZE analyses were compared with those obtained by HPLC.

Keywords: Capillary electrophoresis; *Beta vulgaris*; Betalains; Betanin; Isobetanin

1. Introduction

Red beet root (*Beta vulgaris* ssp. *vulgaris* convar. *vulgaris* var. *vulgaris*) (Chenopodiaceae) is used as a vegetable, food colourant and additive to cosmetics [1,2]. Extracts of this plant have been used in folk medicine since ancient times mainly for cancer treatment but also for the therapy of liver, spleen and skin diseases [3]. Today, red beet root extracts form an ingredient of single pharmaceutical preparations which are mainly used in tumour therapy [4–6].

Red beet root contains abundant amounts of betalain pigments which include two main groups of compounds, namely red betacyanins and yellow betaxanthins. Of the betacyanins, 75–95% consist of betanin together with smaller amounts of isobetanin and prebetanin; the main betaxanthin derivatives are vulgaxanthin I and II [7–10]. Betalains are water-soluble N-containing pigments, which are fairly unstable in alkaline solution when exposed to light and oxygen. In solution these pigments may exist as cations or anions, depending on the pH [10].

So far, analyses for betalains have been accomplished by paper electrophoresis and HPLC [5,7,9–12]. The purpose of this study was the development of a capillary zone electrophoretic (CZE) method for the separation and determination of betanin and isobetanin in different extracts of *B. vulgaris*.

2. Experimental

2.1. Materials

Methanol, acetic acid, H_3PO_4, NaOH, Na_2HPO_4 and citric acid were purchased from Merck (Darmstadt, Germany). Betanin and isobetanin (Fig. 1) were isolated from red beet root and the corresponding aglycones betanidin and

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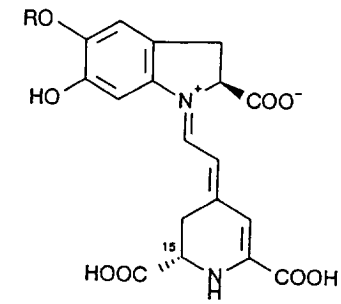
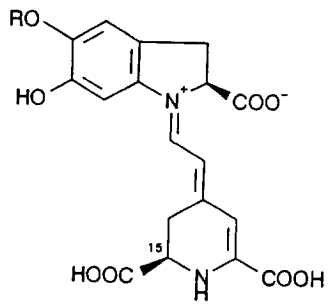


1 R= Glc

3 R= H



2 R= Glc

4 R= H

Fig. 1. Structures of betanin (**1**), isobetanin (**2**), betanidin (**3**) and isobetanidin (**4**).

isobetanidin were obtained by hydrolysis of the glycosides. Lyophilized red beet extract (powder, batch No. 2817) (sample A) and Anthozym-Petrasch-Saft (batch No. 51629) (sample B) were obtained from Petrasch (Dornbirn, Austria); fresh red beet juice was prepared from fresh root material, which was cut into small pieces, homogenized in an Ultra-Turrax mixer and filtered (sample C). Biotta-Randensaft (Biotta) (sample D) was purchased in a grocer's shop in Innsbruck (A).

2.2. Isolation of betanin and isobetanin

Lyophilized red beet material (sample A, 2 g) was subjected to RP-18 chromatography (Li-Chroprep RP-18, 40–63 μm; Merck) using 0.05 *M* acetic acid–methanol (80:20) as the mobile phase. Fractions of 3 ml were collected. Fractions containing pure betanin and isobetanin were combined and evaporated to dryness and the residue was dissolved in a small amount of water and freeze-dried, yielding 5 mg of each compound.

2.3. Sample preparation

Betanin was dissolved in water in order to obtain final solution concentrations of 0.002–0.070 mg/ml for CZE and 0.001–0.400 mg/ml for HPLC analyses. Lyophyilized red beet extract (50 mg) (sample A) was dissolved in 10.00 ml of water. Samples B, C and D were diluted with water in a ratio of 1:10.

2.4. CZE analyses

CZE experiments were performed with a Spectra Phoresis 1000 system (Thermo Separation Products, Fremont, CA, USA) equipped with an high-speed scanning UV–Vis detector, an automatic injector, a temperature-controlled column cartridge with a fused-silica capillary (67 cm × 75 μm I.D.), an autosampler and a printer. The detection wavelengths were 538 and 477 nm, respectively. All experiments were carried out at 15°C at a constant voltage of −22 kV. The running electrolyte was a citrate–phosphate buffer (10 m*M*, pH 6.0).

When studying the effect of the pH of the running electrolyte on the resolution of betanin and isobetanin, pH values between 5.8 and 6.3 were used. Injections were made using the vacuum mode for 3 s each. The pH of each buffer solution was checked with a pH meter. All sample and buffer solutions were filtered through 0.45-μm filters (Sartorius, Göttingen, Germany). Between runs, the capillary was washed with 1% H_3PO_4 at 20°C, followed by equilibration with running buffer (3 min, 15°C). After six runs the capillary was washed with 1 *M* NaOH for 4 min and with 0.1 *M* NaOH for 3 min at 60°C, followed by water (6 min) and buffer (10 min) at 15°C.

2.5. Calibration (CZE)

The calibration graph [$x = 1.35 \cdot 10^{-4}y$, where y = absorbance and x = concentration (mg/ml)]

was obtained using standard solutions containing betanin at concentrations between 0.002 and 0.070 mg/ml (solvent: water).

2.6. HPLC analyses

Analytical HPLC was carried out with a Hewlett-Packard Model 1090 Series II liquid chromatograph on a LiChroCART 125-4 LiChrospher 60 RP-Select B column, particle size 5 μm (Merck). Gradient elution from 2 to 30% B in 30 min was applied, where A = 0.1% phosphoric acid and B = MeOH at a flow-rate of 1 ml/min. The injection volume was 20 μl and UV detection was performed at 477 and 538 nm.

2.7. Calibration (HPLC)

The calibration graph [$x = 0.0152y$, where y = absorbance and x = concentration (mg/ml)] was obtained using standard solutions containing the betanin at concentrations between 0.001 and 0.400 mg/ml (solvent: water). The correlation factor was higher than 0.9998.

2.8. Spectrophotometric analyses

Aqueous solutions of samples A and D were measured with a Hitachi U-2000 UV–Vis spectrophometer at 538 nm. The pigment content was determined using the molar absorptivity of betanin ($\varepsilon = 65\,000$ [11]).

3. Results and discussion

Optimization of the CZE parameters was carried out by investigating the influence of electrolyte composition, pH, electric field, ionic strength and temperature on the mobilities of the betalains betanin (**1**) and isobetanin (**2**) and the corresponding aglycones betanidin (**3**) and isobetanidin (**4**).

Resolution of the betacyanin pairs betanin–isobetanin and betanidin–isobetanidin at six different pH values of the running electrolyte is shown in Fig. 2. The resolution (R_s) of adjacent pigment pairs obviously increases with increase in pH. However, an increase in pH also results in a drastic increase in the migration times (Fig. 2). An acceptable resolution ($R_s > 1.2$) of adjacent pigment pairs and an acceptable analysis time (<60 min) were observed at pH 6.0. At this pH betalains are negatively charged and migrate to the anode. As a result of reversed polarity (−22 kV) of the electrodes, the electroosmotic flow is directed towards the injection end.

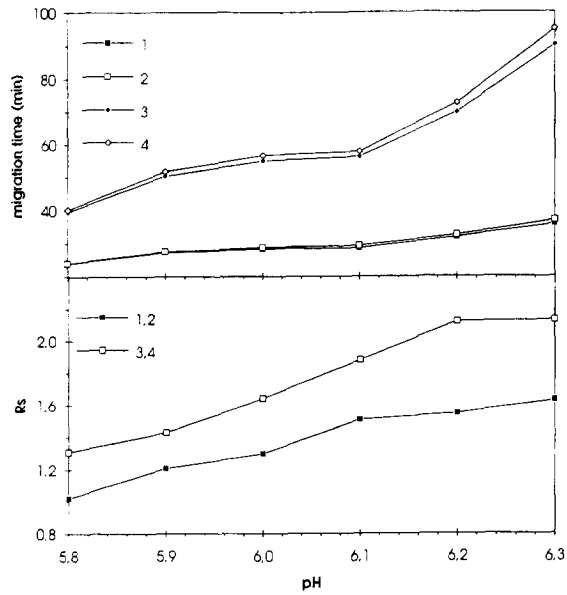


Fig. 2. Effect of pH (5.8–6.3) on resolution (R_s) and migration times of the betacyan pairs **1–2** and **3–4**. Except for pH values, the conditions are as in Fig. 3.

The capillary thermostating temperature was set at 15°C in order to keep degradation of betacyanins as low as possible. The degradation rate of betanin and isobetanin in an aqueous solution of a red beet extract (sample A) was almost twice as high when incubated for 25 h at 25 instead of 15°C.

An electric field strength of −22 kV and an ionic strength of the running electrolyte of 10 m*M* gave satisfactory separations of the *B. vulgaris* pigments. Both parameters had only a slight influence on the resolution of the

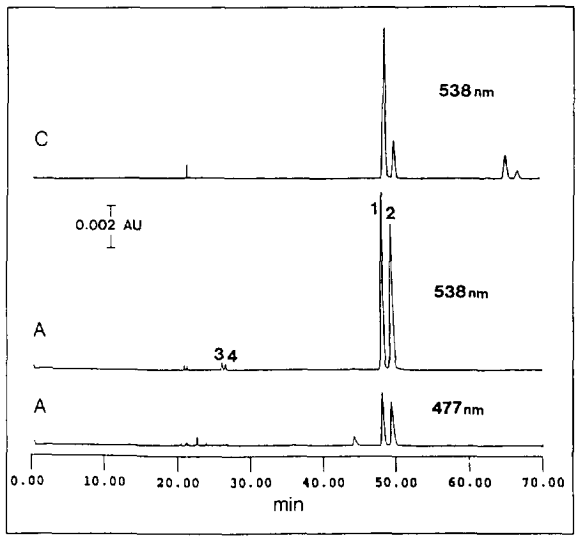


Fig. 3. Electropherograms of samples A and C. Peaks: 1 = betanin; 2 = isobetanin; 3 = betanidin; 4 = isobetanidin. Running electrolyte, 10 m*M* citrate–phosphate buffer (pH 6.0); column, fused silica (67 cm × 75 μm I.D.), injection, vacuum mode, 3 s; voltage, −22 kV; detection, UV at 477 and 538 nm; temperature, 15°C.

betacyanin pairs betanin–isobetanin and betanidin–isobetanidin.

Baseline separation of the four betacyanins could be achieved using a fused-silica capillary tube with 10 m*M* citrate–phosphate buffer (pH 6.0). The applied voltage was −22 kV and the capillary thermostating temperature 15°C. Detection was performed at 477 and 538 nm (Fig. 3).

Determinations of betanin and isotetanin in samples A–D were performed using the external standard method. In the fresh red beet juice (sample C) the betanin concentration is significantly higher than that of isobetanin. In comparison, the pharmaceutical preparation (sample B) and sample D contain similar amounts of the two betacyanins. The aglycones betanidin and isobetanidin are present only in samples A and B and are lacking in the electropherograms of samples C and D.

Calibration was performed with betanin, which is assumed to have a molar absorptivity identical with that of isobetanin [11]. The graph obtained was linear in the range 2–70 μg/ml. The correlation factor was higher than 0.998. The detection limit obtained for betanin and isobetanin was around 1 μg/ml.

The results of quantification are shown in Table 1. The relative standard deviations for quantitative analyses of the samples A–D (five experiments each) were between 2.7 and 5.6% for all compounds studied. The quantitative results obtained are in close agreement with values obtained by HPLC. HPLC analyses were performed using LiChrospher 60 RP-Select-B as stationary phase and a solvent gradient of 0.1% phosphoric acid–methanol (from 2 to 30%

Table 1
Determination of betanin (**1**) and isobetanin (**2**) in samples A–D by CZE, HPLC and spectrophotometry without separation of pigments

Sample	Betanin	Isobetanin	Total betacyans
A	303.3 (3.9)	277.3 (3.6)	580.6 mg per 100 g
	259.4 (0.6)[a]	253.7 (1.1)[a]	513.1 mg per 100 g[a]
	–	–	646.1 mg per 100 g[b]
B	13.0 (3.1)	10.9 (2.7)	23.9 mg per 100 ml
C	33.0 (5.2)	7.2 (5.6)	40.2 mg per 100 ml
D	19.0 (4.4)	15.2 (4.2)	34.2 mg per 100 ml
	17.0 (0.9)[a]	14.1 (0.9)[a]	31.1 mg per 100 ml[a]
	–	–	35.0 mg per 100 ml[b]

Data are means of five replicates with relative standard deviations (%) in parentheses. Values are for CZE except where indicated otherwise.
[a] By HPLC.
[b] By spectrophotometry.

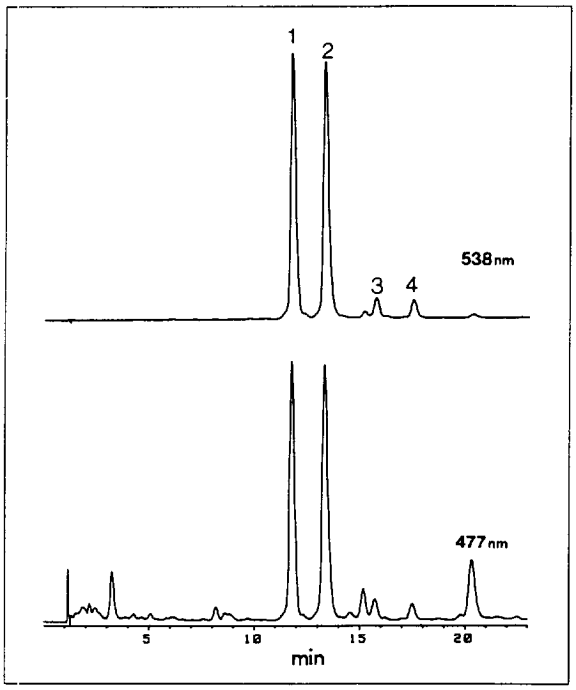


Fig. 4. High-performance liquid chromatograms of sample A. Peaks: 1 = betanin; 2 = isobetanin; 3 = betanidin; 4 = isobetanidin. Column, LiChrospher 60 RP-Select-B; mobile phase, linear gradient of 0.1% phosphoric acid–methanol from 2 to 30% methanol in 30 min; flow-rate, 1 ml/min; injection volume, 20 μl; detection, UV at 477 and 538 nm.

methanol in 30 min) (Fig. 4). The detection limit was ca. 0.7 μg/ml. Quantitative information was obtained using the external standard method (see Table 1, samples A and D). Of course, the total betacyanin content of the two samples determined spectrophotometrically without separation of pigments is significantly higher.

Both HPLC and CZE provide acceptable resolution for the betacyan pairs **1–2** and **3–4** and good reproducibility of retention times. Unfortunately, the analysis time in CZE is much longer than that in HPLC, because the electroosmotic flow in CZE is directed towards the injection end. However, the analysis time in CZE could be dramatically shortend if one could focus only on the separation of the two major red pigments, leaving unseparated the agylcones betanidin and isobetanidin, and also the yellow pigments which are eluted as a number of small signals between 20 and 30 min. A major advantage of CZE is, of course, the low analysis costs. No explanation could be found for the small differences in the quantitative CZE and HPLC results. The peak purities in both techniques were checked and found to be satisfactory.

In conclusion, CZE can be used successfully for the separation and determination of betacyanins from *B. vulgaris* and may be extended to include quantification of the vulgaxanthines, the yellow pigments of red beet root. The betacyanin contents of samples of *B. vulgaris* determined by CZE and HPLC are comparable.

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